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Response Surface Methodology for the Optimization of l-asparaginase Production from isolated *Micrococcus luteus bec 24*

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The main objective of the present study is to optimize the cultural conditions for maximum production of L-Asparaginase from isolated actinomycetes, under solid state fermentation using sesame (black) oil cake (SOC). To determine the effect of five selected process variables (Temperature, pH, Moisture content, Particle size and Inoculum volume) on the production of L-Asparaginase, a central composite design (CCD) coupled with a surface response analysis was used. Empirical quadratic equations were fitted to the experimental data relevant to the yields of L-Asparaginase with five chosen variables. The application of these optimal conditions allowed an increment of 1.81 times in the final L-Asparaginase activity compared with the non-optimized conditions. The combination of conventional method with CCD was effective and reliable in selecting the statistically significant factors and determining the optimal levels of those factors in fermentation medium. The observations made in this study hold great promise for the maximum production of L-Asparaginase using a cheap raw material in SSF by a novel isolate. This work also demonstrates the usefulness of CCD for determining the optimum conditions for the maximum production of L-Asparaginase. To the best of our knowledge, this is the first report on production of L-Asparaginase from *Micrococcus luteus*.

Key words: L-Asparaginase; *Micrococcus luteus*; Sesame (black) oil cake; Solid state fermentation; Central composite design.

L-Asparaginase, the enzyme which catalyses the hydrolysis of asparagine to form aspartic acid and ammonia, is an important natural product that possesses a broad spectrum of anti-tumor activity. It has been a main stay of combination chemotherapy protocols used in treatment of pediatric acute lymphoblastic leukemia (ALL) for almost 40 years[1]. Based on this, it has also been included in most contemporary, multi-agent regimens for adult ALL[2]. L-Asparaginase has been found in various plant and animal species, but due to the difficult extraction procedure of L-Asparaginase, other potential sources like microorganisms were searched[3]. Microorganisms have proved to be very efficient and inexpensive sources of L-Asparaginase. The ease, with which they can be cultivated, has facilitated the large-scale production of the enzyme[4].

A wide range of microbes comprising bacteria, actinomycetes, fungi, yeast and algae are very efficient producers of this enzyme, but enzyme properties vary from organism to organism[5]. Recent

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studies revealed that actinomycetes are proved to be the potential source for L-Asparaginase production. Several *Streptomyces* species such as *S. karnatakensis, S. venezuelae, S. longsporusflavus* and a marine *Streptomyces sp. PDK2*[6], *Streptomyces griseolutueus* WS3/1 [7], *Streptomyces sp* (TA22) [8], *Streptomyces noursei* MTCC 10469 [9] are capable of producing L-Asparaginase.

Solid state fermentation (SSF) systems were termed as a 'low-technology' system from the previous three decades, appear to be a promising ones for the production of value-added 'low volume–high cost' products such as biopharmaceuticals. SSF holds tremendous potential for the production of enzymes. SSF offers numerous advantages over submerged fermentation system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation and simple fermentation equipments. Potentially many high value products, enzymes, primary and secondary metabolites, could be produced in SSF [10]. Agro-industrial residues are generally considered the best substrates for the SSF processes. Bioprocess, utilizing oil cakes is attractive due to relatively cheaper availability of the oil cakes throughout the year, making it even more favorable when economics is considered. Oil cakes are byproducts obtained after oil extraction from the seeds[11].

Screening and evaluation of nutritional and environmental requirements of microorganism is an important step for bioprocess development. As the biological system is a highly complex process, interactions among fermentation factors have enormous impact on cellular metabolism and subsequent product formation. Statistically planned experiments effectively tackle the problem which involves the specific design of experiments which minimizes the error in determining the effect of parameters and the results are achieved in an economical manner. Statistically-based optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model. Several researchers have applied these techniques for optimization[12].

From the foregone discussion it can be concluded that L-Asparaginase is an important drug in treatment of patients with lymphoid malignancies. The demand for L-Asparaginase grew because of its myelo suppressive nature and no late effects caused after the medication and it has become an ideal agent for combination chemotherapy protocol for childhood leukemia. L-Asparaginase can be produced from Plant, animal and microbial source, where plants and animals failed to produce sufficient amounts of L-Asparaginase to meet the present day demands. Considering the therapeutic and commercial importance of L-Asparaginase and lack of suitable high yielding indigenous industrial strains, the present investigation is aimed to isolate a microbial strain to produce high titers of L-Asparaginase and optimize the production of L-Asparaginase using a locally available Agro-industrial waste.

MATERIALS AND NETHODS

Microorganism

An actinomycetes strain was isolated from soil sample collected from a small town named Bapatla in the state of Andhra Pradesh, India and the culture was identified as *Micrococcus luteus* by 16S rRNA analysis. The strain was cultivated on yeast extract-malt extract-dextrose agar medium at 30°C for 7 days [6].

Assay for screening of Microorganism for L-Asparaginase activity

A novel and semi quantitative rapid plate assay for screening of L-Asparaginase producers was adopted[13]. It includes the incorporation of a pH indicator, phenol red in a stock solution that was prepared in ethanol with L-asparagine containing medium. On incubation phenol red appears yellow at acidic pH and turns pink at alkaline pH, thus a pink zone is formed around microbial colonies, which produce L-Asparaginase.

Raw material

Local Sesame (black) oil cake, after extracting the oil from sesame, was collected and grounded to obtain a coarse powder. The coarse powder thus obtained was passed through a sieve to get a homogeneous powder. SOC has protein content of 35% and rich of amino acid profile[11].

Cultivation conditions and crude enzyme extraction

The fermentation medium with the

following composition, 10 g of SOC (0.2cm particle size) and 5 ml of 0.1 M Sodium phosphate buffer (pH 7.0), was prepared and dispensed in 250 ml Erlenmeyer flasks. Unless otherwise indicated, the flasks were autoclaved for 15 min at 121^{0}C and inoculated with 1 ml of the inoculum. The culture flasks were then incubated at 37^{0}C for 96 h without shaking. The extracellular enzyme was isolated at the end of the fermentation period by the addition of 90ml of 0.01M sodium phosphate buffer (pH 7.0) to the medium followed by centrifugation at 8000rpm for 20min. The cell-free supernatant thus obtained was used as crude enzyme preparation[14].

Determination of L-Asparaginase activity

Nesslarization is the most commonly used method for the determination of L-Asparaginase activity. The quantity of ammonia formed during the hydrolysis of 0.04M L-asparagine was estimated using Nessler's reagent in spectrophotometric analysis at 480nm. One unit (IU) of L-Asparaginase activity is defined as the amount of enzyme which liberates 1 ìmol of ammonia per minute. The enzyme activities were estimated in culture filtrates by nesslerization and given in U/gds expression[14].

Optimization by central composite design

The central composite design has been applied to optimize the levels and explain the combined effect of the screened medium constituents at central values, viz., Temp, pH, Moisture Content, Particle Size, and Inoculum volume on the production of L-Asparaginase from isolated *Micrococcus luteus*. Each variable (effective component) was assessed at five coded levels (-2, -1, 0, +1, and +2) with 44 (=2^k+2k+2) treatment combinations where k is the number of independent variables[15]. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form. The quadratic model for predicting the optimal levels was expressed according to the following Equation

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^{\,2} + \Sigma\beta_{ij} X_i X_j \quad ...(1)$$

Where Y is the predicted response, X is the coded levels of the independent variables, β_o is the offset term, âi is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient. The statistical software package STATISTICA 8.0 (Evaluation version) was used for the regression analysis of the experimental data.

RESULTS AND DISCUSSION

Thirty two species were isolated from the locally collected soil samples and checked for their L-Asparaginase activity. Finally one species was chosen based on good zone of color variation among the isolated species. The selected strain was identified as *Micrococcus luteus* based on Macro morphology (colony characters) and Micro morphology (using light microscopy) and 16s rDNA sequence and designated as *Micrococcus luteus BEC 24*. L-Asparaginase production potential of *Micrococcus luteus BEC 24* (Isolated) was compared with the four L-Asparaginase producing cultures (*Escherichia coli* (ATCC 4157), *Erwinia chrysanthemi* (ATCC 1163), *Serratia marcescens* (NCIM 2919), *Pseudomonas aeruginosa* (NCIM 5210) available in India). Among the five cultures tested for the production of L-Asparaginase, isolated *Micrococcus luteus BEC 24* has shown relatively more enzyme production in solid and liquid media (Table 1). Locally available different Agro-industrial residues, Sesame (white) oil cake, Sesame (black) oil cake,

Table 1. Screening of microorganisms for the production of L-Asparaginase in SmF

S. No	Microorganism	Medium	L-Asp activity (IU/ml)
1	*Escherichia Coli*(ATCC 4157)	Modified	21.4385
2	*Erwinia crysanthemi*(ATCC 1163)	M9 medium	27.1735
3	*Serratia marcescens*(NCIM 2919)		37.653
4	*Pseudomonas aeruginosa*(NCIM 5210)		46.0871
5	*Micrococcus luteus BEC 24*		49.2921

Table 2. Experimental design along with experimental and CDD predicted L-Asparaginase activity

Run No	Temp (^{0}C)	pH	Moisture Content (%w/w)	Particle (mm)Size	Inoculum (% V/W) Volume	L-Asparaginase Activity (U/dgs) Experimental	Predicted
1	13.2	7	160	12.5	15	1846.37	1878.35
2	20	6	140	10	10	739.85	779.38
3	20	6	140	10	20	876.52	852.08
4	20	6	140	14	10	1240.47	1168.79
5	20	6	140	14	20	1292.25	1265.79
6	20	6	180	10	10	1260.89	1208.54
7	20	6	180	10	20	1305.54	1323.35
8	20	6	180	14	10	1272.33	1341.33
9	20	6	180	14	20	1626.34	1480.44
10	20	8	140	10	10	765.29	738.35
11	20	8	140	10	20	828.23	798.53
12	20	8	140	14	10	1189.55	1170.51
13	20	8	140	14	20	1231.49	1254.99
14	20	8	180	10	10	1023.43	959.82
15	20	8	180	10	20	1056.93	1062.11
16	20	8	180	14	10	1012.05	1135.36
17	20	8	180	14	20	1258.30	1261.95
18	25	4.7	160	12.5	15	878.40	1004.88
19	25	7	112.5	12.5	15	839.85	910.22
20	25	7	160	7.7	15	871.59	968.69
21	25	7	160	12.5	3.1	374.17	332.18
22	25	7	160	12.5	15	1833.95	1846.89
23	25	7	160	12.5	26.9	413.90	518.60
24	25	7	160	17.3	15	906.64	890.87
25	25	7	207.6	12.5	15	928.41	921.05
26	25	9.4	160	12.5	15	904.31	849.83
27	30	6	140	10	10	1043.05	1003.97
28	30	6	140	10	20	1152.77	1027.96
29	30	6	140	14	10	1065.31	1057.72
30	30	6	140	14	20	1091.88	1106.01
31	30	6	180	10	10	1074.29	1073.57
32	30	6	180	10	20	1169.00	1139.67
33	30	6	180	14	10	914.76	870.70
34	30	6	180	14	20	923.62	961.10
35	30	8	140	10	10	1060.76	1146.33
36	30	8	140	10	20	1206.89	1157.81
37	30	8	140	14	10	1251.29	1242.83
38	30	8	140	14	20	1284.01	1278.60
39	30	8	180	10	10	1019.51	1008.25
40	30	8	180	10	20	1027.26	1061.83
41	30	8	180	14	10	823.81	848.13
42	30	8	180	14	20	979.40	926.01
43	36.9	7	160	12.5	15	1629.89	1645.53
44	36.9	7	160	12.5	15	1629.89	1645.53

Table 3. Estimated regression coefficients for the L-Asparaginase activity

Constant	Coeff.	t-value	p-value
Mean/Interaction	-35524.5	-11.850	0.000
X_1	212.1	4.504	0.000
X_2	2440.3	8.455	0.000
X_3	191.1	12.415	0.000
X_4	1406.9	10.992	0.000
X_5	301.1	7.175	0.000
X_1^2	-0.6	-0.955	0.349
X_2^2	-166.3	-9.735	0.000
X_3^2	-0.4	-9.864	0.000
X_4^2	-39.8	-9.976	0.000
X_5^2	-10.0	-15.056	0.000
$X_1 X_2$	9.2	3.288	0.003
$X_1 X_3$	-0.9	-6.446	0.000
$X_1 X_4$	-8.4	-6.077	0.000
$X_1 X_5$	-0.5	-0.873	0.392
$X_2 X_3$	-2.6	-3.723	0.001
$X_2 X_4$	5.3	0.772	0.448
$X_2 X_5$	-0.6	-0.224	0.824
$X_3 X_4$	-1.6	-4.638	0.000
$X_3 X_5$	0.1	0.755	0.458
$X_4 X_5$	0.6	0.439	0.665

Soy bean meal, Groundnut oil cake, Coconut oil cake, Black gram husk, Sunflower oil cake, Rice flour, Ragi meal, Banana fruit peel, Banana fruit stalk, Rice bran, Saw dust, Green gram husk and Green pea husk were screened for the production of L-Asparaginase by isolated *M.leteus.* Among different solid substrates screened for the production of L-Asparaginase, SOC produced maximum enzyme activity when incubated with the isolated microorganism

Identification of the isolated Microorganism

To determine the phylogenetic position of isolate, the 16S rDNA sequence was determined and a phylogenetic tree was created by comparison of the new sequence with other known relevant sequences in the GenBank database. The tree clearly indicated that isolated strain and the other known species of genus *Micrococcus* should be grouped into the same lineage. The apparent closest relative to strain *Micrococcus luteus BEC 24* was *Micrococcus luteus*, with a sequence similarity of 94±4%; this branch received 100% bootstrap support. Based on nucleotides homology, phylogenetic analysis, nearest homolog species, the isolated organism was found to be *Micrococcus luteus* (AJ717367) (Fig 1) [16].

Microbes represent a potential source for commercially important bioactive compounds. Among microorganisms, actinomycetes have gained special importance as the most potent source of antibiotics and other bioactive secondary metabolites. While most of the studies on actinomycetes have focused on antibiotic production, only few reports have delt on their enzymatic potential. Likewise actinomycetes are providing good L-asparaginase enzyme when compared to bacterial and fungal sources. Isolate used in this study was unique with other actinomycetes studied in terms of production capabilities and operation and genetic stability etc.

Production of L-Asparaginase

Preliminary studies on L-Asparaginase production with *Micrococcus luteus BEC 24* in SSF was performed using various locally available agricultural materials. Among selected materials, Sesame oil (Black) cake supported the maximum Asparaginase production (Fig 2), indicating the importance of nature of agro-material in enzyme production by this isolate. This data suggested that the isolated bacterial strain of *Micrococcus luteus BEC 24* has higher potential in L-Asparaginase production. Conventional one-at-a-time experimental procedure revealed that five independent variables such as Temperature, pH, Moisture content, Particle size, and Inoculum volume were the most influential factors for this enzyme production with Sesame oil (Black) cake as solid medium.

Optimization by central composite design

In the optimization of bioprocess variables for enzyme production by the organism, the combination of conventional method with central composite design (CCD) is effective and reliable in selecting the statistically significant factors and determining the optimal concentration of those factors in fermentation medium [7]. This work demonstrates the usefulness of CCD for determining the optimum conditions for maximizing L-Asparaginase production. In order to approach the optimum response region of the L-Asparaginase activity, significant independent variables (Temperature, X_1; pH, X_2; Moisture content, X_3; Particle size, X_4; and Inoculum volume X_5), were further explored, each at five levels. Table 2 represents the design matrix of the variables in

natural units together with the experimental results of L-Asparaginase activity. The L-Asparaginase production varied from 374.17U/gds to 1846.37U/gds and an average response of 1093.74U/gds was obtained, indicating the influence of selected parameters levels role on *Micrococcus luteus BEC 24* metabolism and subsequent L-Asparaginase production. An excellent correlation between predicted and measured values of these experiments justifies the validity of response model and the existence of an optimum point.

To understand an empirical relationship between the L-Asparaginase activity (Y) and test variables and also to perform the analysis of

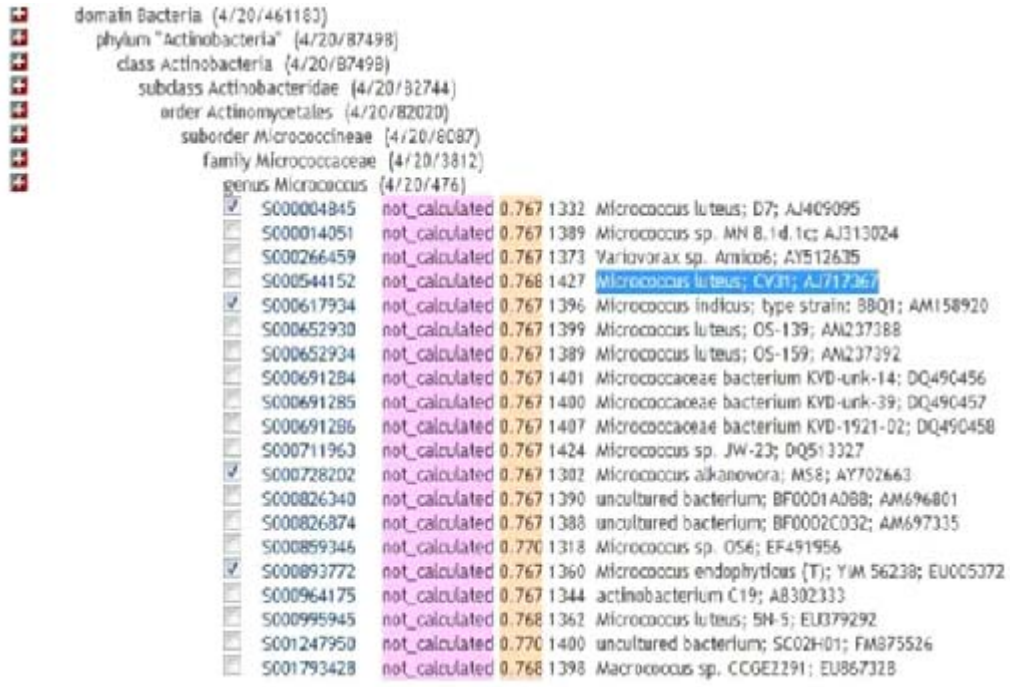

Fig. 1. Species identification based in 16srRNA sequence comparision

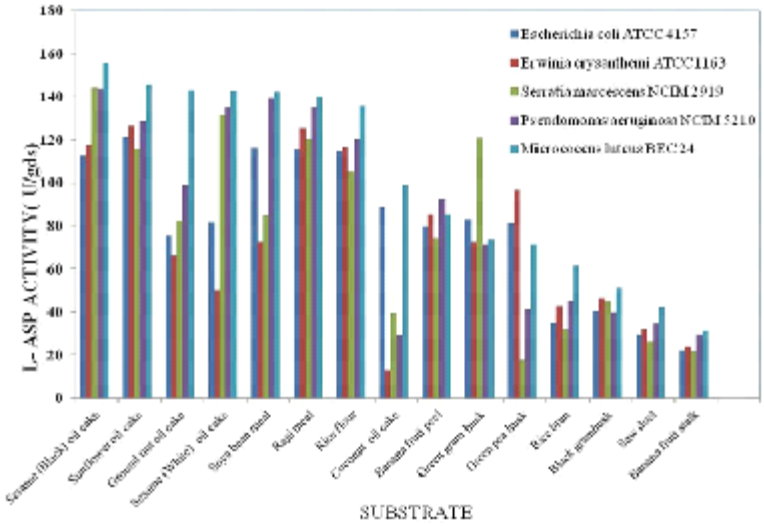

Fig. 2. Screening of different substrates with different microorganisms for the production of L-asparaginase

variance (ANOVA), the data was analyzed using second order surface model using following Equation.

$$Y = -35524.5 + 212.1X_1 + 2440.3X_2 + 191.1X_3 + 1406.9X_4 + 301.1X_5 + 9.2X_1X_2 - 0.9X_1X_3 - 8.4X_1X_4 - 0.5X_1X_5 - 2.6X_2X_3 + 5.3X_2X_4 - 0.6X_2X_5 - 1.6X_3X_4 + 0.1X_3X_5 + 0.6X_4X_5 - 0.6X_1^2 - 166.3X_2^2 - 0.4X_3^2 - 39.8X_4^2 - X_5^2$$

...(2)

Where Y is L-Asparaginase activity in U/gds; X_1, X_2, X_3, X_4 and X_5 Temperature, pH, Moisture content, Particle size, and inoculum volume respectively.

The predicted L-asparaginase activity resulted from equation (2) are in close agreement with the experimental values as evident from last column of Table 2, and hence the above equation was deemed to be adequate in representing the solid state fermentation of L-asparaginase production under the specified range of experiments. At the model level, the correlation measures for the estimation of the regression equation and the determination coefficient R^2. The closer the value of R^2 is to 1, the better is the correlation between the observed and the predicted values[12]. In this experiment, the values of R^2 were 0.965 for L-Asparaginase activity indicating 96.5% of the variability in the response could be explained by the model. The value of adjusted determination coefficient (Adj. $R^2 = 0.934$) is also very high which indicate a high significance of the model. These values indicate a high degree of correlation between the experimental and the predicted values. The parity plot (Figure 3) showed a satisfactory correlation between the experimental and predicted values of L-asparaginase activity, wherein, the points cluster around the diagonal line which indicated the good fit of the model because the deviation between the experimental and predicted values is less.

The regression model data in the form of response surface plots revealed that L-Asparaginase production was highly and interactively influenced by all selected. Response surface plots as a function of two factors at a time, maintaining all factors at a fixed levels (zero, for instance) are more helpful in understanding both the main and the interaction effect of the two factors. These plots can be easily obtained by calculating from the model, the values taken by one factor where the second varies (from -2 to +2) with constant of a given Y value. The surface plot is not only describing the individual effect of variables but also cumulative effect of these two test variables on the response. The activity value for different concentrations of the variable can also be predicted from the respective response surface plots[17].

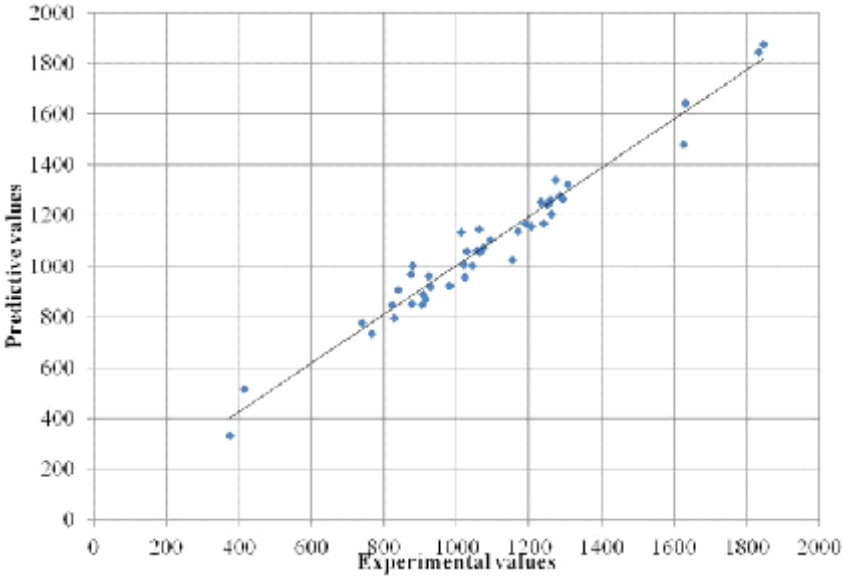

Fig. 3. Parity plot showing the distribution of experimental Vs predicted values of L- asparaginase activity

The analysis revealed a maximum activity of L-Asparaginase by isolate *Micrococcus luteus BEC 24* was of 1782.38U/ gds. The critical levels of the five variables as obtained from the maximum point of the polynomial model were estimated using the STATISTICA, and found to be: Temperature (38.3^0C), pH (7.4), Moisture content (146.76%w/w), Particle size (11.29mm) and Inoculum volume (14.95%v/w). Experiments in triplicate were carried out at the above optimized conditions and an average response of 1730.72U/gds was obtained which was quite closer to the optimal value predicted by the Central Composite Design.

Yasser and Zakia (2002), reported maximal L-Asparaginase activity (142.8 IU) using Box-Benhken design with pH 7.9; casein hydrolysate, 3.11%; and corn steep liquor, 3.68% which is more than five folds the activity in basal medium by *Pseudomonas aeruginosa* in solid-state culture[18]. Hymavathi et al., used fractional factorial central composite design (FFCCD) under SSF using Red gram husk for the optimization of L-Asparaginase production by isolated *Bacillus circulans* MTCC 8574. L-Asparaginase yield improved from 780 to 2,322 U/gds which is more than 300% [19]. Sita and Narasimha., applied Doehlert experimental design (DD) for the optimization of L-Asparaginase production by *Yarrowia lipolytica* NCIM 3472 in solid state fermentation (SSF) using palm kernel cake. The maximum enzyme activity obtained was 39.8623 U/gds at the optimal set of conditions: moisture content of the substrate: 54.8622 (%), sodium citrate concentration: 11.9241 (%w/w) and L-asparagine concentration: 1.0758 (%w/w)[20]. Kamala et al., optimized four process parameters for the production of L-Asparaginase using CCD from *Steptomyces griseoluteus*. 24.56 IU/ml was found to be the maximum enzyme activity with the optimal points of dextrose, L-asparagine, pH, inoculum at 0.45%w/v, 1.162%w/v,7.4, 10.36%w/v respectively[7].

Overall, our results indicate that the isolate *Micrococcus luteus BEC 24* revealed higher enzyme production compared to those reported in the literature. Optimization studies showed nearly a twofold improvement in L-Asparaginase production using SSF. Each selected fermentation factor showed its influence at individual or interactive level either in linear or quadratic terms. The study emphasizes the selection of different process parameter levels that would influence economic production of L-Asparaginase production

The present study used the Central composite design (CCD) to optimize medium constituents of the fermentation medium for the production of L-Asparaginase from Sesame oil cake by SSF using isolated *Micrococcus luteus BEC 24*. Five variables viz., Temperature, pH, Moisture Content, Particle Size and Inoculum volume were identified by preliminary experimental runs as significant for L-Asparaginase activity. These variables are optimized with CCD using STATISTICA 8.0 for the necessary computations. All the five variables showed significant influence on the L-Asparaginase activity. The significant interactions between the five variables were also observed from the response surface and contour plots (Table 3). The maximum activity of L-Asparaginase produced from Sesame cake was predicted to be 1782.38U/gds. The high L-Asparaginase activity achieved in conjunction with the abundantly available Sesame cake (in the state of Andhra Pradesh, India) paved a way for the industrial exploitation of this substrate under solid state fermentation using the indigenous isolated *Micrococcus luteus* as a suitable microorganism.

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